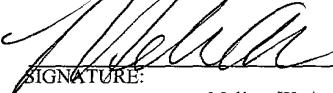


FORM PTO 1390 (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER HO-P02300US0
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>09/937533</b>
INTERNATIONAL APPLICATION NO. PCT/EP00/02632	INTERNATIONAL FILING DATES 24/03/2000			PRIORITY DATE CLAIMED 24 March 1999
TITLE OF INVENTION SURFACE AND ITS MANUFACTURE AND USES				
APPLICANT(S) FOR DO/EO/US Anders Larsson, et al.				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing 35 U.S.C. 371</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371 (f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c)(2))        a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).        b. <input type="checkbox"/> has been communicated by the International Bureau.        c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).        a. <input type="checkbox"/> is attached hereto.        b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))        a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).        b. <input checked="" type="checkbox"/> have been communicated by the International Bureau.        c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.        d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</p>				
<b>Items 11 to 20 below concern document(s) or information included:</b>				
<p>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Fee Transmittal; PCT Written Opinion; Notification of the Recording of a Change; International Preliminary Examination Report; International Search Report and Response to Written Opinion</p>				

JC09 Rec'd PCT/PTO 24 SEP 2001

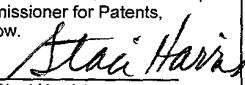
U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
<b>09/937533</b>	PCT/EP00/02632	HO-P02300US0
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY
<b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) – (5)):</b>		
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1000.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00		
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		
Surcharge of <u>\$ 130.00</u> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		
CLAIMS	NUMBER FILED	NUMBER EXTRA
Total claims	29-20 =	9
Independent claims	4-3 =	1
MULTIPLE DEPENDENT CLAIM(s) (if applicable)		x
<b>TOTAL OF ABOVE CALCULATIONS =</b>		
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.		
<b>SUBTOTAL =</b>		
Processing fee of \$ for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +		
<b>TOTAL NATIONAL FEE =</b>		
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31) + \$ ( per property).		
<b>TOTAL FEES ENCLOSED =</b>		
<b>Amount to be Refunded:</b> \$ <b>Charged:</b> \$		
a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>06-2375</u> in the amount of \$ <u>616.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to my Deposit Account No. <u>06-2375</u> . A duplicate copy of this sheet is enclosed.		
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.</b>		
SEND ALL CORRESPONDENCE TO: Melissa W. Acosta FULBRIGHT & JAWORSKI L.L.P. 1301 McKinney, Suite 5100 Houston, Texas 77010-3095 (713) 651-5407 Customer Number: 26271		
 SIGNATURE: <u>Melissa W. Acosta</u> NAME: _____ 45,872 REGISTRATION NUMBER: _____		

09/937533

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I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EK102655026US, in an envelope addressed to: Box Non-Fee Amendment, Commissioner for Patents, Washington, DC 20231, on the date shown below.

Dated: September 24, 2001

Signature:   
(Staci Harris)

Docket No.: HO-P02300US0  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:  
Anders Larsson, et al.

Application No.: Not Yet Assigned

Group Art Unit: N/A

Filed: September 24, 2001

Examiner: Not Yet Assigned

For: SURFACE AND ITS MANUFACTURE AND  
USES

**FIRST PRELIMINARY AMENDMENT**

**Box Non-Fee Amendment**  
Commissioner for Patents  
Washington, DC 20231

Dear Sir:

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

**In the Claims**

Please cancel claims 9 and 14. Please substitute the following amended claims contained herein for claims 1-8, 10-13 and 15-22 that were in the amended PCT application. Applicants have included in Appendix A, a marked version of the claims to illustrate the changes contained herein.

1. (Amended) A method for rendering a substrate surface containing a channel with a depth < 1000 $\mu$ m intended to form a liquid transportation system and being made in plastic material more hydrophilic comprising:

treating the surface with a gas plasma of a non-polymerizable gas, wherein the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic.

2. (Amended) The method according to claim 1, wherein the plasma intensity is  $\geq 5 \text{ W/cm}^3/\text{min}$ .

3. (Amended) The method according to claim 2, wherein a power of  $> 250 \text{ W}$  and a gas flow of  $< 50 \text{ cm}^3/\text{min}$  are applied to create the plasma.

4. (Amended) The method of claim 1, wherein the plastic material comprises an immediate water-contact angle  $> 20^\circ$  and the plasma treatment conditions are set so that the immediate water-conduct angle after plasma treatment and a subsequent wash with pure water/ethanol becomes  $< 30^\circ$ .

5. (Amended) The method of claim 1, wherein the plastic material is a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups.

6. (Amended) The method of claim 1, wherein the plasma is induced by radiowaves, microwaves, or a combination thereof.

7. (Amended) The method of claim 1, wherein the plasma gas is selected from the group consisting of oxygen, nitrogen, noble gas, or a mixture thereof.

8. (Amended) The method of claim 1, wherein subsequent to the treating step, the surface of the substrate is derivatized to exhibit anion exchanging groups, cation exchanging groups, amphoteric groups, hydroxy groups, bioaffinity groups, or chelating groups.

10. (Amended) A substrate surface, which is made of a plastic material, which has been plasma treated, comprising a surface in uncoated form having an immediate water-contact angle of < 30°, wherein said water-contact angle is changed less than + 20% and/or less than + 5° upon washing with a pure ethanol/water mixture.

11. (Amended) The substrate of claim 10, wherein the plastic material is a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups.

12. (Amended) The substrate of claim 10, wherein the surface before having been gas plasma treated exhibits an immediate water-contact angle > 30°.

13. (Amended) The substrate of claim 10, wherein at least part of the surface comprises a liquid transportation system comprising a channel which has a depth < 1000 µm.

15. (Amended) A kit comprising

a microfabricated device comprising a substrate surface which is made in a plastic material and which comprises a liquid transportation system comprising a channel which has a depth < 1000 µm, and

a fluorescent substance to be detected in the device, the fluorescence intensity of the plastic material being < 50 % of the fluorescent intensity of the substance at the wavelength at which substance fluoresces.

16. (Amended) The kit of claim 15, wherein the plastic comprises a polymerisation product obtained by polymerisation of an aliphatic monomer in which there is polymerisable unsaturation.

17. (Amended) A method for culturing anchorage-dependent cells and non-anchorage dependent cells that in a part of their life cycle require attachment to a substrate surface comprising performing the culturing of the cells in contact with a substrate surface which is made of a plastic material and has with an immediate water-contact angle of < 30°.

18. (Amended) The method of claim 17, wherein the substrate surface having an immediate contact angle of  $\leq 30^\circ$  has been obtained by the gas plasma treatment method defined in claim 1.

19. (Amended) The method of 17, the substrate surface enables at least 30 % of the plated anchorage dependent cells to adhere to the substrate surface.

20. (Amended) The method of claim 17, wherein at most 15 % of the culture medium is serum.

21. (Amended) The method of claim 17, wherein culturing is taking place during a time period permitting the number of cells to be at least duplicated.

22. (Amended) The method claim 17, wherein the cell culturing is carried out in a chamber of a microfabricated device in which there is a liquid transportation system comprising a channel having a depth which is  $< 1000 \mu\text{m}$  and a chamber which provides said substrate surface.

Please add the following new claims.

23. The method according to claim 1 further comprising the step of washing the surface subsequently with a pure solvent selected from the group consisting of water, a water-miscible solvent and mixtures thereof.

24. The method according to claim 5, wherein said polymer is a copolymer.

25. The method according to claim 5, wherein said unsaturated monomer(s) is an alkene/alkadiene or a vinyl aryl compound.

26. The method according to claim 25, wherein said alkene/alkadiene is selected from the group consisting of acids, esters, amides, and nitriles containing one or more alkene groups.

27. The substrate of claim 10, wherein said polymer is a copolymer.

28. The substrate of claim 11, wherein said unsaturated monomer(s) is an alkene/alkadiene or vinyl aryl compounds.

29. The substrate of claim 28, wherein said alkene/alkadiene is selected from the group consisting of acids, esters, amides, and nitriles containing one or more alkene groups.

30. The substrate of claim 11, wherein said polymer is cross-linked.

31. The substrate of claim 11, wherein said polymer is a mixture of two or more polymers or copolymers.

## REMARKS/ARGUMENTS

Claims 1-22 were in the original PCT application as filed. Applicants have amended claims 1-8, 10-13 and 15-22 to delete the multiple dependency and to clarify the claims without prejudice or acquiescence. Claims 9 and 17 have been canceled without prejudice or acquiescence. Applicants have also added new claims 23-31, which relates to the subject matter that was contained in the multiple dependent claims of the PCT application. Applicants have included a marked up version of the claims as amended herein as Appendix A. For the convenience of the Examiner, Applicants have included in Appendix B a copy of all pending claims as amended herein. Applicants assert that no new matter has been added.

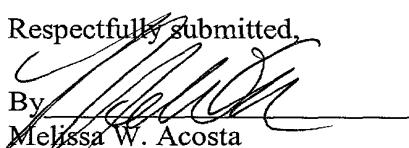
## CONCLUSION

Claims 1-22 were in the amended PCT application. Applicants have amended claims 1-8, 10-13 and 15-22 to delete the multiple dependency and to clarify the claims. Claims 9 and 17 have been canceled. New claims 23-31 have been added, which are related to the subject matter in the original multiple dependent claims. Therefore, these amendments do not narrow the scope of the claims within the meaning of *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co., Ltd.*, 234 F.3d 558, 586, 56 USPQ2d 1865, 1886 (Fed. Cir. 2000).

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

Dated: September 24, 2001

Respectfully submitted,

By   
Melissa W. Acosta

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## APPENDIX A

**Version With Markings to Show Changes Made**

1. (Amended) A method for rendering a substrate surface containing a channel with a depth  $< 1000\mu\text{m}$  intended to form a liquid transportation system and being made in plastic material more hydrophilic comprising:

~~by treatment treating the surface with in a gas plasma of a non-polymerizable gas, characterized in that wherein the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic, and with the optional step that the surface subsequently is washed with a pure solvent selected from water, a water-miscible solvent and mixtures thereof, said surface containing a liquid transportation system in which there is a channel with a depth  $\leq 1000\mu\text{m}$ .~~

2. (Amended) The method according to claim 1, characterized in that wherein the plasma intensity is  $\geq 5 \text{ W/cm}^3/\text{min}$ .

3. (Amended) The method according to claim 2, characterized in that wherein a power of  $\geq 250 \text{ W}$  and a gas flow of  $\leq 50 \text{ cm}^3/\text{min}$  are applied to create the plasma.

4. (Amended) The method of any one of claims 1-3, characterized in that wherein the plastic material ~~has been~~ is selected among plastics having comprises an immediate water-contact angle  $\geq > 20^\circ$  and (a) the plasma treatment conditions are set so that the immediate water-conduct angle after plasma treatment and a subsequent wash with pure water/ethanol (~~70% w/w~~) becomes  $\leq < 30^\circ$ .

5. (Amended) The method of any one of claims 1-4, characterized in that wherein the plastic material is a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups. ~~s/copolymers in which the monomer/ is unsaturated such as (i) alkenes/alkadienes, and acids, esters, amides, nitriles etc containing one or more alkene groups; and (ii) vinyl aryl compounds in which the vinyl group is bound to aryl that optionally is substituted with lower alkyl groups (C1-6); (b) condensation~~

~~polymers/copolymers in which the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc. groups.~~

6. (Amended) The method of ~~any one of claims 1-5~~ 1, characterized in that wherein the plasma is induced by radiowaves ~~and/or~~ microwaves, or a combination thereof.

7. (Amended) The method of ~~any one of claims 1-6~~ 1, characterized in that wherein the plasma gas is selected from the group consisting of oxygen, nitrogen, a noble gas, such as argon, and/or a mixture thereof ~~these gases~~.

8. (Amended) The method of ~~any one of claims 1-7~~ 1, characterized in that wherein subsequent to the plasma treatment the treating step, the surface of the substrate is derivatized to exhibit ~~groups selected amongst~~ anion exchanging groups, cation exchanging groups, amphoteric groups, hydroxy groups, bioaffinity groups, and/or chelating groups.

9. canceled

10. (Amended) A substrate surface, which is made of a plastic material, which has been plasma treated, comprising a characterized in that the surface in uncoated form has having an immediate water-contact angle of  $\leq < 30^\circ$ , wherein said water-contact angle is being changed less than  $\pm 20\%$  and/or less than  $\pm 5^\circ$  upon washing with a pure ethanol/water mixture (70% w/w).

11. (Amended) The substrate of claim 10, characterized in that wherein the plastic material is selected from (a) a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups, ~~copolymers in which the monomers are unsaturated such as (i) alkenes/alkadienes, and acids, esters, amides, nitriles etc containing one or more alkene groups; and (ii) vinyl aryl compounds that optionally is substituted with lower alkyl groups (C1-6); or condensation polymers/copolymers, in which the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc. groups; said polymer material optionally being cross-linked and/or optionally being is a mixture of two or more kinds of polymers/copolymers.~~

12. (Amended) The substrate of ~~any one of claims 10-11~~ 10, characterized in that wherein the surface before having been gas plasma treated exhibits an immediate water-contact angle  $\geq 30^\circ$ .

13. (Amended) The substrate of ~~any one of claims 10-12~~ 10, wherein at least part of the surface comprises a liquid transportation system comprising a channel which has a depth  $\leq 1000 \mu\text{m}^{\text{**}}$ .

14. canceled

15. (Amended) A kit characterized in comprising

(a) a microfabricated device comprising a substrate surface which is made in a plastic material and which comprises a liquid transportation system comprising a channel which has a depth  $\leq 1000 \mu\text{m}^{\text{**}}$ , and

(b) a fluorescent substance to be detected in the device, the fluorescence intensity of the plastic material being  $< 50\%$  of the fluorescent intensity of the substance at the wavelength at which substance fluoresces.

16. (Amended) The kit of claim 15, characterized in that wherein the plastic comprises a polymerisation product obtained by polymerisation of an aliphatic monomer in which there is polymerisable unsaturation.

17. (Amended) A method for culturing anchorage-dependent cells and non-anchorage dependent cells that in a part of their life cycle require attachment to a substrate surface comprising performing the culturing of the cells in contact with a substrate surface which is made of producing characterized in that the substrate surface is made in a plastic material and has with an immediate water-contact angle of  $\leq 30^\circ$ .

18. (Amended) The method of claim 17, characterized in that wherein the substrate surface having an immediate contact angle of  $\leq 30^\circ$  has been obtained by the gas plasma treatment method defined in claims 1-9.

19. (Amended) The method of any one of claims 17-18 17, with the provison that for anchorage dependent cells the substrate surface enables at least 30 % of the plated anchorage dependent cells to adhere to the substrate surface.

20. (Amended) The method of any one of claims 17-19 17, characterized in that wherein at most 15 % of the culture medium is serum.

21. (Amended) The method of any one of claims 17-20 17, characterized in that wherein culturing is taking place during a time period permitting the number of cells to be at least duplicated.

22. (Amended) The method of any one of claims 17-21 17, characterized in that wherein the cell culturing is carried out in a chamber of a microfabricated device in which there is a liquid transportation system comprising (a) a channel having a depth which is  $\leq$  1000  $\mu\text{m}^{\text{#}}$  and (b) said a chamber which also provides said substrate surface.

APPENDIX B

PENDING CLAIMS AS OF SEPTEMBER 24, 2001

1. A method for rendering a substrate surface containing a channel with a depth  $< 1000\mu$  intended to form a liquid transportation system and being made in plastic material more hydrophilic comprising:

treating the surface with a gas plasma of a non-polymerizable gas, wherein the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic.

2. The method according to claim 1, wherein the plasma intensity is  $\geq 5 \text{ W/cm}^3/\text{min}$ .

3. The method according to claim 2, wherein a power of  $> 250 \text{ W}$  and a gas flow of  $\leq 50 \text{ cm}^3/\text{min}$  are applied to create the plasma.

4. The method of claim 1, wherein the plastic material comprises an immediate water-contact angle  $> 20^\circ$  and the plasma treatment conditions are set so that the immediate water-conduct angle after plasma treatment and a subsequent wash with pure water/ethanol becomes  $< 30^\circ$ .

5. The method of claim 1, wherein the plastic material is a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups.

6. The method of claim 1, wherein the plasma is induced by radiowaves, microwaves, or a combination thereof.

7. The method of claim 1, wherein the plasma gas is selected from the group consisting of oxygen, nitrogen, noble gas, and a mixture thereof.

8. The method of claim 1, wherein subsequent to the treating step, the surface of the substrate is derivatized to exhibit anion exchanging groups, cation exchanging groups, amphoteric groups, hydroxy groups, bioaffinity groups, or chelating groups.

9. canceled

10. A substrate surface, which is made of a plastic material, which has been plasma treated, comprising a surface in uncoated form having an immediate water-contact angle of < 30°, wherein said water-contact angle is changed less than + 20% and/or less than + 5° upon washing with a pure ethanol/water mixture.

11. The substrate of claim 10, wherein the plastic material is a polymer comprising unsaturated monomer(s) or condensation polymer(s), wherein said condensation polymer(s) comprises monomer(s) having two or more groups selected from the group consisting of amino groups, hydroxy groups and carboxy groups.

12. The substrate of claim 10, wherein the surface before having been gas plasma treated exhibits an immediate water-contact angle > 30°.

13. The substrate of claim 10, wherein at least part of the surface comprises a liquid transportation system comprising a channel which has a depth < 1000 µm.

14. canceled

15. A kit comprising

a microfabricated device comprising a substrate surface which is made in a plastic material and which comprises a liquid transportation system comprising a channel which has a depth < 1000 µm, and

a fluorescent substance to be detected in the device, the fluorescence intensity of the plastic material being < 50 % of the fluorescent intensity of the substance at the wavelength at which substance fluoresces.

16. The kit of claim 15, wherein the plastic comprises a polymerisation product obtained by polymerisation of an aliphatic monomer in which there is polymerisable unsaturation.

17. A method for culturing anchorage-dependent cells and non-anchorage dependent cells that in a part of their life cycle require attachment to a substrate surface

comprising performing the culturing of the cells in contact with a substrate surface which is made of a plastic material and has with an immediate water-contact angle of < 30°.

18. The method of claim 17, wherein the substrate surface having an immediate contact angle of ≤ 30° has been obtained by the gas plasma treatment method defined in claim 1.

19. The method of 17, the substrate surface enables at least 30 % of the plated anchorage dependent cells to adhere to the substrate surface.

20. The method of claim 17, wherein at most 15 % of the culture medium is serum.

21. The method of claim 17, wherein culturing is taking place during a time period permitting the number of cells to be at least duplicated.

22. The method claim 17, wherein the cell culturing is carried out in a chamber of a microfabricated device in which there is a liquid transportation system comprising a channel having a depth which is < 1000 μm and a chamber which provides said substrate surface.

23. The method according to claim 1 further comprising the step of washing the surface subsequently with a pure solvent selected from the group consisting of water, a water-miscible solvent and mixtures thereof.

24. The method according to claim 5, wherein said polymer is a copolymer.

25. The method according to claim 5, wherein said unsaturated monomer(s) is an alkene/alkadiene or a vinyl aryl compound.

26. The method according to claim 25, wherein said alkene/alkadiene is selected from the group consisting of acids, esters, amides, and nitriles containing one or more alkene groups.

27. The substrate of claim 10, wherein said polymer is a copolymer.

28. The substrate of claim 11, wherein said unsaturated monomer(s) is an alkene/alkadiene or vinyl aryl compounds.

29. The substrate of claim 28, wherein said alkene/alkadiene is selected from the group consisting of acids, esters, amides, and nitriles containing one or more alkene groups.

32. The substrate of claim 11, wherein said polymer is cross-linked.

33. The substrate of claim 11, wherein said polymer is a mixture of two or more polymers or copolymers.

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SURFACE AND ITS MANUFACTURE AND USES

The present invention concerns a method for enhancing the hydrophilicity of a polymer surface by treatment with a plasma.

5 A common method for surface modification of plastics is to subject them to various forms of plasma treatment (Chan et al., Surface Science Reports 24 (1996) 1-54; and Garbassi et al., Polymer Surfaces - From Physics to Technology, John Wiley (1998) 238-241). This is done in a plasma reactor, which is a  
10 vacuum vessel with a gas at low pressure (typically 10 to 1000 mTorr). When a high frequency electric field is applied over the reactor, a plasma (also called glow discharge) is formed, containing reactive species like ions, free radicals and vacuum-UV photons. These species react with the plastics  
15 surface and cause a chemical modification with properties depending on the nature of the gas and on the plasma parameters. Gases like oxygen and argon are typically used for hydrophilisations and adhesion improvement on nonpolar plastics, while vapours of polymerising monomers can be used  
20 to apply thin coatings on plastics for a number of different purposes (Yasuda, Plasma Polymerization, Academic Press 1985).

There are a number of publications on treatment of polycarbonate surfaces with oxygen and argon plasmas. A  
25 stability study (Morra et al, Angew. Makromol. Chem. 189(3184) (1991) 125-136) showed that much of the hydrophilicity of the treated surfaces was lost after either water extraction or 3 days' dry storage. The hydrophilicity loss after water extraction was due to the formation of low  
30 molecular weight water-soluble surface species during the plasma treatment. The storage instability was attributed to rearrangement of the polymer chains in the surface. An ESCA study (Greenwood et al., Macromolecules 30 (1997) 1091-1098) showed that 79% of the oxygen incorporated in the  
35 polycarbonate surface by oxygen plasma treatment was removed by washing with a 1:1 cyclohexane/isopropanol mixture. This

GY0018 PCT (former PU9909)

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is attributed to degradation of polymer chains during the plasma treatment.

Similar effects have also been observed for polystyrene. An ESCA-study of a plasma-treated tissue-culture polystyrene 5 showed about 35% loss of surface oxygen after water washing (Onyiriuka et al., J. Coll. Interf. Sci. 144(1) (1991) 98). In two other ESCA studies, oxygen-plasma treated polystyrene gave 25% surface oxygen loss after water washing (Callen et al., J. Vac. Sci. Technol. A 13(4) (1991) 2023-2029), (Morra 10 et al., Angew. Macromol. Chem. 189 (3184) (1991) 125-136). A polystyrene surface treated with an oxygen plasma had initially a water-contact angle of 7°, but after a methanol wash the contact angle increased to 64° (Murakami et al., J. Coll. Interf. Sci. 202 (1998) 37-44).

15 WO 9618498 describes an attempt to produce a permanently hydrophilised surface made of plastics. The method comprises a first step in an inorganic gas plasma in order to introduce charges on the surface and a second step during which a polyionic polymer having the opposite charge is adsorbed to 20 the surface.

EP-A-106,046 describes hydrophilisation of fluorinated polymer surfaces by treatment in a gas plasma in which the main component is a polymerising nitrogen-containing organic compound.

25 GB 2,061,969 describes the manufacture of hydrophilic and antistatic vinyl chloride polymer by treatment in an inorganic gas plasma. The problem of rendering the plastics permanently hydrophilic is not mentioned.

DE 3712491 describes gas plasma hydrophilization of porous 30 membranes made of various synthetic polymers. Plastic material is not mentioned. Storage stability of the hydrophilized membranes is indicated but there is no discussion related to stability during washing conditions in aqueous milieu. Liquid contact angles are measured, but since

porous surfaces typically gives significantly decreased angles compared to no-porous smooth surfaces, these values cannot simply be compared with the values given in this specification.

5 EP 106662 describes a microtiter plate made of a dark plastic material in order to improve fluorescence measurements in the wells. Microtiter plates are normally devoid of liquid transportation systems in microformat.

The electric excitation field applied typically has a  
10 frequency in the radiowave or microwave region, i.e. kHz-MHz or GHz respectively. The modification (hydrophilisation) on the polymer surface caused by the plasma will depend mainly on a number of internal plasma parameters such as: type of species present in the plasma, spatial distributions, energy  
15 distributions and directional distributions. In turn these parameters depend in a complex way on the external plasma parameters: reactor geometry, type of excitation, applied power, type of process gas, gas pressure and gas flow rate.

In many applications involving contact between polar  
20 liquids and surfaces it is of no big concern whether an introduced hydrophilicity is stable towards washing or not. Particular problems are encountered in case the polymer surface is part of a channel of capillary dimensions, where a high degree of hydrophilicity is necessary if aqueous liquids  
25 are to be introduced by self-suction or by centripetal forces. This becomes particularly true in case a repeated contact is to take place reproducibly, in which case an unstable surface modification will be washed away during the first liquid contact. The smaller dimensions of the channel  
30 the more severe the problem becomes.

In the context of the invention the expression "plasma treated surface" will, if not otherwise specified, refer to an uncoated naked plasma treated surface, possibly being

GY0018 PCT (former PU9909)

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derivatized to contain separate reactive species firmly bound to the surface.

- 5 Cell culturing in microfabricated devices has been described previously in for instance WO 9955827 with priority from April 27, 1998.

WO 9721090 relates to a microfluidic device in which centrifugal force is used to drive the liquids. In one 10 sentence it is suggested that microculture and identification of pathogens can be made within the device without any hint at the selection of the proper surface characteristics.

Cell aggregates have previously been cultured in vessels with a water contact angle below 30° (JP patent application 15 19930119579, Derwent abstract accession number 1995-047885.

Adherence of cells to gas plasma treated polytetrafluoro ethylene (PTFE) surfaces with water contact angles 20-45° has been studied (Dekker et al., Clinical Materials 11 (1992) 157-162). Adherence appears to have required abnormally high 20 concentrations of substances promoting adherence (20 % human serum-containing culture medium) compared to the most commonly used 10 % or less.

Adherence of CHO cells to surfaces which have been gas plasma hydrophilised in the presence of H<sub>2</sub>O-vapour has been 25 studied in order to look for optimal cell cultivation properties of plastic surfaces (Lee et al., Biomaterials

12(5) (1991) 443-448). Poor cell adherence to the most hydrophilic surfaces was found found.

Microfluidic devices in which liquid transportation systems are defined by hydrophilic/hydrophobic barriers have been described previously in for instance WO 9958245 with priority from May 8, 1998.

Experimental results partly corresponding to this invention have been presented at the Second International Symposium on Polymer Surface Modification, New Ark June 1999 (Anders 10 Larsson: Plasma Treated Polycarbonate as Substrate for Culture of Adherent Mammalian Cells).

#### Objectives of the invention

- A first objective is to provide a gas plasma method 15 for hydrophilisation of polymer surfaces, which enhances the stability of the hydrophilicity introduced.
- A second objective is to provide plasma treated surfaces that are hydrophilic after the treatment and 20 remain so upon repeated wetting/drying, i.e. have an initial hydrophilicity that is not significantly altered in contact with hydrophilic liquids, for instance ethanol/water mixtures.
- A third objective is to provide capillary/channel/ 25 chamber system; for instance in microfabricated form, having capillaries/channels/chamber, the inner surfaces of which being as defined for the second objective and permitting repeated introduction of aqueous solutions in a reproducible way.
- A fourth objective is to provide liquid 30 transportation systems in which at least a part of the inner surfaces complies with the second objective.
- A fifth objective is to provide plasma treated 35 surfaces that can be used for cell culturing, assay reactions etc.

These objectives are mainly adapted to surfaces that before the plasma treatment have a relatively high immediate water-contact angle, for instance  $\geq 20^\circ$ , such as  $\geq 30^\circ$  or even  $\geq 50^\circ$ . By the term "immediate water-contact angle" is meant 5 that the contact angle is measured on a dry surface before an applied liquid has significantly evaporated. See the experimental part.

#### The invention

10 We have now discovered that the objectives given above can be met, if the polymer surface (plastics surface) is brought into contact with a gas plasma of high intensity (energy input per gas molecule). Our discovery is explainable in terms of two types of polar groups being introduced: (1) 15 Groups retained firmly on the polymer surface and (2) groups allocated to loosely held degradation fragments. The first alternative will result in a stable hydrophilicity. The second alternative will result in a hydrophilicity, which is easily removed by contact with polar liquids, such as aqueous 20 solutions.

Accordingly a first aspect of the invention is a method for rendering a polymer surface (plastics surface) permanently more hydrophilic by contacting the surface with a gas plasma so that firmly bound polar groups are introduced on the 25 surface. Most likely these polar groups are introduced directly on the polymer skeleton constituting the surface, possibly involving cross-linking of the surface layer.

Hydroxy and or amino groups, carboxy groups, ether groups etc and other groups in which a carbon atom binds to a 30 heteroatom selected among oxygen, sulphur, and nitrogen are examples of polar groups that may be introduced. Changes in surface presence of this-type of groups may be studied by ESCA (XPS).

35 The expressions "permanently more hydrophilic" and "stable hydrophilicity" contemplate that the immediate water-contact

angle remains essentially unchanged upon washing with ethanol (70 % w/w, washing procedure as given in the experimental part). This means that the washing procedure should not be allowed to change the immediate water-contact angle more than  $\pm 20\%$  and/or more than  $\pm 5^\circ$ .

The storage stability (in dry form) of the hydrophilised surface should be at least one month with acceptable increases in immediate water-contact water angle not being larger than  $10^\circ$ , preferably not larger than  $5^\circ$ . In case the storage stability in dry form is not acceptable, sufficient storage stability often can be accomplished by storing in aqueous atmosphere or in an aqueous liquid.

The method of the invention may have an optional washing step subsequent to the gas plasma treatment step. This washing procedure means contacting the gas plasma treated surface with an aqueous solution or some other polar liquid to remove loosely held hydrophilic compounds. The washing solution is preferably water, a water-miscible liquid or a mixture of these. Examples of water-miscible liquids are methanol, ethanol, isopropanol, n-propanol, t-butanol, sec-butanol, dimethyl formamide, dimethyl sulphoxide, acetone and other liquid compounds having similar solubilities in water.

The required intensity of the plasma will depend on the variables discussed above. Satisfactory gas plasmas may be found in case the electric excitation power applied is  $\geq 250$  W with preference for  $\geq 300$  W, and typically  $500 \pm 100$  W with a gas flow selected in the interval of  $\leq 50$  cm<sup>3</sup>/min, with preference for  $\leq 25$  cm<sup>3</sup>/min. For the plasma intensity, the interval is normally  $\geq 5$  W/cm<sup>3</sup>/min, such as  $\geq 10$  W/cm<sup>3</sup>/min or  $\geq 20$  W/cm<sup>3</sup>/min or even  $\geq 35$  W/cm<sup>3</sup>/min. Normalised values per m<sup>2</sup> electrode area will typically be  $\geq 30$  W/m<sup>2</sup>/cm<sup>3</sup>/min, such as  $\geq 60$  W/m<sup>2</sup>/cm<sup>3</sup>/min or  $\geq 120$  W/m<sup>2</sup>/cm<sup>3</sup>/min or even  $\geq 215$  W/m<sup>2</sup>/cm<sup>3</sup>/min. The pressures are typically lower than 100

mTorr, with preference for pressures that are  $\leq$  50 mTorr. These ranges apply for a temperature of 25°C, atmospheric pressure and oxygen. For other gases the values must be multiplied with  $M_{O_2}/M_x$ , where  $M_{O_2}$  and  $M_x$  are the molecular weights of oxygen and the other gas, respectively.

The gases used should be non-polymerisable in the type of plasma contemplated. Typical such gases are inorganic. This means that suitable gases are found among oxygen, nitrogen, noble gases (such as helium, neon, argon, krypton, xenon) and mixtures thereof, such as air and mixtures containing other proportions of oxygen and nitrogen. Other potentially useful gases are carbon dioxide, carbon monoxide, water vapour etc that might be used either solely or in combination. By varying the composition of gas the groups inserted onto the surface it is likely that the groups can be varied with respect to kinds and density.

Illustrative examples of polymerisable gases are volatile lower organic molecules such as lower hydrocarbons and vapours of allyl- or acryl monomers, aromatics etc. An inorganic non-polymerisable gas may be present together with a polymerisable organic compound in gas form.

Plasma reactor vessels enabling a sufficiently high power output combined with proper gas flow velocities are commercially available. As stated above the reactor vessels should enable an electric excitation power input for instance in the microwave or radio wave ranges. A suitable plasma reactor is PS0500 (BOC Coating Technology, USA) which permits a radiofrequency (RF) power of 0-500 W and gas flow of 0-100 or 0-1000 standard cm<sup>3</sup>/min.

The results of a treatment may depend on the design of the reactor vessel used meaning that the optimal interval to a certain degree will vary from one reactor design to another. The results may also depend on where in the reactor the surface is placed during the treatment.

The immediate water-contact angle of the polymer material (plastics) covering the surface to be gas plasma treated is typically  $\geq 20^\circ$ . The largest advantages with the invention are obtained for polymer materials that provide larger immediate water-contact angles, such as  $\geq 30^\circ$  for instance  $\geq 50^\circ$ . These figures refer to plastic surfaces having been cleaned with respect to water-soluble compounds and low molecular weight compounds (typically  $\leq 1$  kD). Illustrative examples of how the water-contact angle may vary with polymer are given in table 1.

The inventive method typically results in an increase in the permanent hydrophilicity which corresponds to lowering the immediate water contact angle of the initial plastics surface more than 20 %, such as more than 50 % (after removal of water-soluble compounds, low molecular weight compounds and the like). A large increase in hydrophilicity is more important for hydrophobic than for hydrophilic plastics.

TABLE 1.

20 Plastic	Water-contact angle
PTFE (Teflon)	108°
Silicone rubber (Sylgard 184)	106°
Polypropylene	95°
25 Polyethylene	94°
Polystyrene	90°
Polycarbonate	78°
PET (polyester)	76°
Styrene-acrylonitrile	73°
30 PMMA (Plexiglas)	59°

Typically the polymer on the surface has been obtained by polymerisation of monomers comprising unsaturation, such as

in carbon-carbon double bonds or carbon-carbon-triple bonds. The polymer may be a homopolymer or a copolymer.

The monomers may, for instance, be selected from mono-, di and poly/oligo-unsaturated compounds, e.g. vinyl compounds and other compounds containing unsaturation. The monomers may or may not contain halogen substituents, such as fluoro and/or chloro. Illustrative monomers are:

- (i) alkenes/alkadienes (such as ethylene, butadiene, propylene and including substituted forms such as 10 vinyl ethers), cycloalkenes, monofluorovinyl and di- and polyfluorovinyl hydrocarbons (for instance tetrafluoroethylene), alkene-containing acids, esters, amides, nitriles etc for instance various methacryl/acryl compounds; and  
15 (ii) vinyl aryl compounds (such as mono-, di- and trivinyl benzenes) that optionally may be substituted with for instance lower alkyl groups (C1-6) etc.

Another type of polymers are condensation polymers in which 20 the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc groups (so called polyfunctional compounds). Particularly emphasised monomers are polyamino monomers, polycarboxy monomers (including corresponding reactive halides, esters and 25 anhydrides), poly hydroxy monomers, amino-carboxy monomers, amino-hydroxy monomers and hydroxy-carboxy monomers, in which poly stands for two, three or more functional groups. Polyfunctional compounds include compounds having a functional group that is reactive twice, for instance 30 carbonic acid or formaldehyde. The polymers contemplated are typically polycarbonates, polyamides, polyamines, polyethers etc. Polyethers include the corresponding silicon analogues, such as silicone rubber.

35 The polymers may be in cross-linked form.

The polymer on the surface may be a mixture of two or more different polymer(s)/copolymer(s). In this case the water-contact angles and their changes discussed above apply to these mixtures, i.e. the factual surface.

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Particularly interesting polymers are those that have a non-significant fluorescence for excitation wavelengths in the interval 200-800 nm and emission wavelengths in the interval 400-900 nm. By non-significant fluorescence is meant 10 that the fluorescence intensity in the above-given emission wavelength interval should be below 50 % of the fluorescence intensity for a reference plastics (= a polycarbonate of bisphenol A without fluorescent additives). In fact it does not harm in case the fluorescence intensity of the plastics 15 is even lower, such as < 30 % or < 15 %, such as < 5 % or < 1 %, of the fluorescence intensity of the reference plastics. Typical plastics having an acceptable fluorescence are polymers of aliphatic monomers containing polymerisable carbon-carbon double bonds, such as polymers of cykloalkenes 20 (e.g. norbornene and substituterade norbornenes), ethylene, propylenes etc, as well as other non-aromatic polymers of high purity, e.g. certain grades of polymethylmethacrylate.

The requirement for a low fluorescence is of particular importance in case the plastics are to be used for carrying 25 samples in which one or more fluorescent substances are to be detected/ measured. It then becomes important to select plastics with non-significant fluorescence at the wavelength at which a fluorescent substance to be detected/measured fluoresces. In most cases this means that the fluorescence 30 intensity of the plastics at the emission wavelength of the substance should be below 50 % of the fluorescence intensity of the substance. In fact it will not harm in case the fluorescence intensity of the plastics is < 30 %, such as < 15 %, or still lower, such as < 5 % or < 1 %, of the 35 fluorescence intensity of the substance. In case the fluorescence of several substances emitting light at

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different wave-lengths are to be measured it will put harsher demands on the plastics, since fluorescence of the plastics then should be non-significant for broader wave-length bands or for several bands.

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After the plasma treatment, optionally after a washing procedure to remove loosely held hydrophilic compounds, the surface may be further derivatized to exhibit one or more type of reactive groups, i.e. groups that are able to bind 10 other compounds either via some type of affinity or via covalent linking. Derivatization preferably takes place after the washing step and may be preceded by a coating step, for instance, to provide the surface with a coating carrying additional hydrophilic groups that can be used for 15 derivatization.

By masking certain parts of the surface and leaving other parts unmasked before hydrophilisation, hydrophilic patterns on a hydrophobic surface can be obtained. Alternatively a 20 hydrophobic pattern may be printed on the surface after the hydrophilisation. These techniques may be of value in the manufacture of microfabricated liquid transportation systems. See below.

25 Various methods for introducing reactive groups on polymers exhibiting hydrophilic groups, such as hydroxy, amino or carboxy etc groups are well known to the averaged skilled artisan in the field. Well known affinity groups are charged groups, and groups exerting affinity via interactions of 30 other types, possibly in combination with charge-charge interactions. Illustrative examples of charged groups are ion-exchanging groups, such as anion and cation exchanging groups, with typical examples being ammonium ions (primary, secondary, tertiary and quaternary ammonium ions), sulphates, 35 sulphonates, phosphates, phosphonates etc. Illustrative examples of other affinity groups are so called bioaffinity

groups including individual members of ligand - receptor pairs, such as antibody - antigen/hapten, complementary nucleic acids, Ig binding proteins - Ig (e.g. protein A or G - IgG), lectins - carbohydrate structures, cells - cell attachment molecules (fibronectin, collagen, RGD-peptides) etc. Included in bioaffinity groups are also semi- and fully synthetic ligands that more or less completely mimics native bioaffinity.

10 The hydrophilised surface may be part of arrangements of different physical forms. The hydrophilised surface may be the bottoms/walls of microtiter wells and other types of vessels and also the outer surfaces of porous and non-porous particle material. The hydrophilised surface may be part of a 15 less hydrophilic larger surface (e.g. a hydrophobic surface).

The hydrophilised surface may provide a significant part of the liquid contact surfaces in systems intended for transport of aqueous liquids. These systems may have channels that may be of capillary dimensions, for instance with a distance 20 between two opposite walls being  $\leq 1000 \mu\text{m}$ , such as  $\leq 100 \mu\text{m}$ , or even  $\leq 10 \mu\text{m}$ , such as  $\leq 1 \mu\text{m}$ . These systems may also contain one or more chambers connected to the channels and having volumes being  $\leq 500 \mu\text{l}$ , such as  $\leq 100 \mu\text{l}$  and even  $\leq 10 \mu\text{l}$  such as  $\leq 1 \mu\text{l}$ . The depths of the chambers may typically 25 be in the interval  $\leq 1000 \mu\text{m}$  such as  $\leq 100 \mu\text{m}$  such as  $\leq 10 \mu\text{m}$  or even  $\leq 1 \mu\text{m}$ . The lower limit is always significantly greater than the largest of the reagents used. The lower limit is typically in the range 0.1-0.01  $\mu\text{m}$  for devices that are to be delivered in dry form. One or more liquid 30 transportation systems of this type may be placed on a common plate, for instance spinnable, such as a disc of CD-type. In the case of spinnable forms, the liquid may be forced through one or more segments of the transportation system by spinning the disc (centripetal force). In this latter case the liquid 35 transportation systems are placed radially. Other types of

pressure generating systems may also be used for transport of liquid in the liquid transportation systems discussed above.

A device having one or more liquid transportation system comprising channels and chambers with a depth  $\leq 1000 \mu\text{m}$ , such as  $\leq 100 \mu\text{m}$  or even groudner than  $10 \mu\text{m}$ , such as  $\leq 1 \mu\text{m}$ , are further on called a microfabricated device. The chambers/channels are said to be in the microformat. A microfabricated device typically has its channels and chambers in one plane, such as in the surface of a plate, for instance on a disc.

10 The plate may be circular, oval, rectangular or of any other 2D geometric form.

The channels and/or chambers are defined by liquid barriers, which are to guide a liquid flow. The liquid barriers can be in form of physical walls, bottoms and tops.

15 Walls in form of hydrophobic barriers for guiding aqueous liquids and in form of hydrophilic barriers for guiding non-polar liquids have been suggested (WO 9955827 with priority from April 27, 1998). By covering a surface (I) to be hydrophilised with a mask leaving a pattern of communicating 20 lines and dots unmasked and hydrophilise, for instance according to the present invention, the surface (I) will exhibit a hydrophilic pattern. When placing a hydrophobic surface (II) (cover or top) against the hydrophilic pattern and leave a capillary slot between the surfaces, a liquid 25 transportation system will be obtained. Surface (II) may also have a hydrophilic pattern matching the hydrophilic pattern of surface (I). As discussed above the hydrophilic pattern may also be obtained by hydrophilising the full surface and then print the desired hydrophobic pattern thereon. The 30 top/cover will prevent evaporation of liquid. It may have minor parts/dots in form of through-passing holes intended for addition/removal of liquids.

An advantageous way of attaching the top/cover to the hydrophobic surface is by thermogluing as described in SE 35 application 0000300-4, filed on January 30, 2000 (which is hereby incorporated by reference). The top/cover should also

allow for gas exchange between the cultivation chamber and ambient atmosphere.

Liquid transportation systems of the type referred to above may also contain valves, pumps, filters and the like.

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The surface may be used for performing chemical reactions of inorganic and/or organic/biochemical nature. The surface may be used as carrier matrix in chromatography, for cell culture, for solid phase chemical synthesis of  
10 oligo/polypeptides, oligo/polynucleotides, other organic polymers and other organic compounds. Illustrative examples of reactions to be run on the surface of the invention are conventional chemical reactions or reactions that are based on affinity involving recognition through geometric fitness  
15 and interactions based on hydrogen-bonding, van-der Waals bonding, dipole-dipole interaction, charge-dipole interaction, charge-charge interaction etc. Vessels having interior surfaces being treated according to the invention may be used for storage of various types of organic and  
20 inorganic chemicals and/or liquids. For cell culturing, further details are given below.

Advantageous further developments of surfaces obtained by the present invention are described in International Patent Application PCT/EP99/10347 (which is hereby incorporated by reference). In these developments the inventive plasma hydrophilization has been applied to part of or to the complete liquid transportation system. After hydrophilization, polyethylene imine to which monomethoxy polyethylene glycol chains are bound is adsorbed to the treated surface. Preferred designs of liquid transportation systems are also described.

A second aspect of the invention is a naked plasma treated  
35 polymer surface (plastics surface) permanently hydrophilised

as defined above and complying with anyone of the other above-mentioned features, either alone or in combination.

A third aspect of the invention is the various uses  
5 discussed above or below of surfaces obtained in accordance  
with the invention and/or having any of the features  
discussed herein and achievable through the inventive  
hydrophilisation method.

10 **Kit containing a microfabricated device**

A fourth aspect is a kit containing

(a) a microfabricated device comprising a liquid  
transportation system in which there are at least one  
chamber and/or at least one channel the walls of which  
15 comprises a synthetic polymer (plastics), and

(b) a fluorescent substance to be detected in the device,  
The kit is characterized in that the synthetic polymer  
material has a fluorescence that is non-significant in the  
same sense as discussed previously in this specification. The  
20 surfaces in the channels and chambers of the microfabricated  
device may wholly or partly be hydrophilised, for instance by  
gas plasma treatment, preferably according to the method  
described herein. With respect to the chemical composition of  
the plastics of the walls (surfaces) and its physical  
25 parameters, the same material with the same preferences as  
given above may be used.

Alternative hydrophilisation protocols are treatment with  
oxidating acids and with UV-oxidations, corona treatment,  
grafting and conventional coating with a polymer providing an  
30 increased number of polar groups, etc on the liquid contact  
surface of the material. The polar groups referred to are for  
instance hydroxy, amino, carboxy, amido, polyethylene oxide  
etc.

Illustrative examples of fluorescent substances are  
35 fluorophores of organic or inorganic origin. In the former  
case they often have a low molecular weight (typically < 1

kD). Important fluorophors have distinct emission wavelengths with distinct maxima in the interval 400-750 nm, with preference for the interval 480-670 nm. Fluorescein, phycocyanines that may be native or chemically modified, 5 rhodamine, Texas Red, fluorescent rare earth chelates (in particular europium and terbium), cadmium selenide nanoparticles etc are typical examples. When in use the fluorophores may be in conjugate form, i.e. covalently attached to a reagent used. In this form the fluorescent 10 substance typically has a molecular weight > 1 kD. The kit, in particular the chambers and the channels combined with the fluorescent substance, may be used for running chemical reactions, assays, separations, cell culturing and the like 15 as described elsewhere in this specification.

15

#### A microfabricated device.

A fifth inventive aspect is a microfabricated device as defined above, in which the liquid transportation system is formed in/on a polymer material (plastics) in which the 20 plastics essentially consist of one or more polymers obtained by polymerising one or more aliphatic monomers of the kind defined above. In this aspect the device may have one or more of the features of the other inventive aspects described in this specification. The same uses apply.

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#### Cell culturing

A sixth inventive aspect is a method for culturing cells. The term culturing of cells as used herein includes monolayer culture, suspension culture etc, and excludes culturing of 30 cell aggregates, tissues, biopsies etc. Cell culturing as contemplated herein encompasses inherent normal cell culturing practice, for instance

- (a) The number of cells should be at least duplicated or at least triplicated during the cultivation period.
- 35 (b) Cultivation of anchorage-dependent cells, which represent an inhomogeneous phenotypic population,

should take place under a low selection pressure for cell adherence to the surfaces used. This implies that for anchorage dependent cells the cell surface should be selected so that at least 30 % of the plated cells should adhere to the substrate surface. More preferably, this adherence percentage should be above 50 % or higher, such as at least 90 %.

(c) In order to promote interaction between cells and substrate surfaces, cell adhesions factors are typically present in the culture medium. For mammalian adherent cells the culture medium typically contain up to 15 % (w/w) serum according to well-established practice.

Culturing of cells, in particular anchorage-dependent cells, have previously been carried out in the presence of substrate surfaces made of plastics. The immediate water-contact angle has been 40°-60°. For cell culturing in microfabricated devices, there will be problems with liquid fluidics with this relatively low hydrophilicity.

It has now been found that culturing of various kinds of cells can be carried out in contact with superhydrophilic substrate surfaces made of plastics having an immediate water-contact angle that is significantly lower than 40°-60°. When applied to microfabricated devices this discovery will improve the situation with respect to liquid fluidics. The sixth aspect thus is characterized in that the cell culturing takes places in the presence of a surface made of plastics providing an immediate water-contact angle  $\leq 30^\circ$ , such as  $\leq 20^\circ$ . The surfaces are primarily provided on the inner walls of the culture vessel, but may also be provided by e.g. particles suspended in the vessel.

The immediate water-contact angle refers to hydrophilicities that preferably are stable against repeated washing as described above.

Surfaces that have been hydrophilised by gas plasma treatment as described in this specification are preferred.

The preferred plasmas contain one or more gases that solely or in combination can introduce the mix of charged/polar groups discussed below. Among the gases tested, mixtures of oxygen and nitrogen are most preferred, with the individual gases being less preferred and with argon being least preferred. One can envisage that gases, such as sulphur dioxide and diphosphorous pentoxide, might be beneficial to use together with oxygen and/or nitrogen, if disregarding the handling problems they might give.

- 10 The most important factor for successful culture and behaviour of anchorage-dependent cells is the surface on which the cells grow. If this type of cells are plated onto a surface to which they cannot adhere or adhere poorly they will not grow. Cell function requires dynamic interactions 15 between the cell and its substratum. These interactions occur at specialised contact sites where transmembrane proteins (integrins; Hynes RO, Integrins: versatility, modulation and signalling in cell adhesion. Cell 69: 11-25, 1992) link the interior of the cell with the external substratum. Surface 20 functional groups and their charge character as well as hydrophilicity/hydrophobicity and surface free energy are important factors for cell behaviour (Lee JH et al., Biomaterials 18:351-358. 1997). Amine (ammonium), amide, hydroxyl, carboxyl (carboxylate) and sulphonyl (sulphonate) 25 and sulphate groups at a suitable density are considered as sites for electrostatic interaction with the cell surface or attachment proteins and as mimicking adhesive contacts on extracellular matrix components (Maroudas, J. Theor. Biol. 49 (1975) 417-442; Lee et al., Biomaterials 15 (1994) 704-711; 30 and Lee et al., Biomaterials 18 (1997) 351-358).

Non-anchorage dependent cells often require substrate surfaces during a certain part of their life cycle.

Typically the density of charged groups should be above 1-2 group per  $\text{\AA}^2$ . As a thumb of rule smaller cells, such as HeLa 35 cells (10-20  $\mu\text{m}$ ), require higher densities than larger cells, such as fibroblasts (about 30x100  $\mu\text{m}$ ), lower densities. The

optimal values vary among cell types and may be determined as known in the art.

Cell culturing according to this aspect of the invention applies to a wide variety of cells. The cells may be 5 anchorage- or non-anchorage-dependent. They may be of normal or tumour origin and they may be genetically manipulated in culture. They may be derived from mammals, bacteria, fungi (yeast), plants, fish, birds, amphibians, reptiles, etc. With respect to mammalian cells they may derive from any tissue, 10 e.g. epithelial, endothelial, fibroblast, muscle, nerve, pigment, hematopoetic and germ cells.

For each respective kind of cell, the rules for selecting conditions and protocols are in principle the same as for culturing in other vessels and on particles.

15 The polymer surface material should not be toxic to the cells to be cultured. We have, for instance recognized, that gas plasma treated polymers built up of acrylo nitrile monomer or acrylate monomer may be toxic, probably due to degradation of the polymer. This may be circumvented by 20 avoiding these kinds of material or by a proper post treatment of the material before it is used.

The invention will now be illustrated by non-limiting experiments. The invention is further defined in the appended 25 claims that are part of the application text.

## EXPERIMENTAL PART

### Materials

30 Surfaces (discs): Polycarbonate of bisphenol A and polymethylmethacrylate CD blanks, injection molded at Toolex Alpha AB, Sundbyberg, Sweden. Non-patterned CD blanks, injection molded from Zeonex (a cycloolefin copolymer from Nippon Zeon, Japan) or Luran KR2536 (a styrene-acrylonitrile 35 copolymer (SAN) from BASF, Germany) at Åmic AB, Uppsala,

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Sweden). The planar (non-patterned) side of the discs were used in all experiments.

Gases: Oxygen, Argon and synthetic air were from l'Air Liquide, France.

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Plasma reactor: Plasma Science, PS0500 (BOC Coating Technology) main adjustable parameters: Radiofrequency (RF) power 0-500 W and gas flow 0-100 or 0-1000 sccm (standard cm<sup>3</sup>/min)

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Normally the reactor PS0500 is equipped with three electrode plates but after a rebuilding only one plate remained.

#### Methods

15 Washing: Before plasma treatment all discs were immersed in pro analysi isopropanol for 2 min, briefly flushed with 99% ethanol and blown dry with house nitrogen. This was done to remove any release agents, antistatic agents etc which might interfere with the plasma treatments.

20

Plasma treatments: The discs were placed in the plasma reactor in one of two positions; either on a plastic support 20.5 cm from the chamber floor or on glass supports placed on the electrode plate (45 cm from the chamber floor). After 25 evacuation to a base pressure of 60 mTorr, the gas was let in and the gas flow adjusted to the desired level. The RF power was then switched on for the intended time and the reactor chamber was finally vented with ambient air.

30 Contact angle measurements: Directly after treatment, the equilibrium water-contact angle was measured with the sessile drop method on a Ramé-Hart goniometer bench. For each sample six measurements were made (two sides on each of three droplets). Contact angle measurements were also made after 35 the pieces had been immersed for 2 min in 70% ethanol/water and blown dry with house nitrogen. The measurement was made

within 20 s after the liquid had been applied in order to avoid changes in contact angle e.g. due to evaporation of the droplets.

5 Check for introduction of cross-links: The polymer material was dissolved in a suitable solvent for original polymer but not for polymer chains cross-linked during the plasma treatment before and after gas plasma treatments. The presence of any insoluble material after treatment was taken 10 as an indication of introduction of cross-links.

Check for introduction of polar oxygen containing groups:

Preliminary studies by ESCA showed that the pattern of these groups in the surface changed upon gas plasma treatment in a 15 way suggesting an increase in surface bound oxygen. ESCA can be used to determine the various polar/charged groups that may be of importance for cell culturing.

20 Storage study: The plasma treated discs were placed in polystyrene Petri dishes and stored under ambient lab conditions. With regular intervals small pieces were cut off and the immediate water-contact angle measured both directly and after immersion in 70% ethanol/water. The cut-off samples were discarded after measurement.

25

Criteria for acceptance: From the hydrophilicity point of view the preliminary acceptance criterion was that the water-contact angle should be 20° or lower after washing in 70% ethanol.

30

Cell culture:

Methods for evaluating surfaces:

Cell culture was used as a method for evaluation of the plasma-treated surfaces, since cells pose extremely high 35 demands on their substratum.

Pieces of plasma-treated material were placed in multi-well plates, a suspension of cells in culture medium was added to each well and the plates were incubated in a cell culture atmosphere for various times. Cell adhesion, morphology and proliferation were evaluated microscopically and occasionally by the use of immunocytochemistry against cell proliferation markers and adhesive contacts. Preliminary results have revealed that the plasma-treated surfaces can be used for cell culture and that the cells exhibit necessary characteristics for optimal behaviour. Such characteristics include adhesion of nearly all of the plated cells in a evenly pattern, proper cell spreading on the material surface, signs of normal cell motility and cell division. Pathological signs including vacoles, excessive amounts of lysosomal granulae, blebbing or membrane destruction were insignificant. The cell lines tested so far include MRC5 (normal lung fibroblasts), HeLa (cervix carcinoma cells of epithelial-like origin), Chang (hepatoma, liver cells). The cells have been selected so that they will cover a wide range of demands on the surfaces. Non-anchorage dependent cells put very small demands on the surfaces as such. An example of such cells (Raji lymphoma), have been successfully cultured in the presence of the plasma-treated surfaces.

Our results suggest efficient cell culturing properties for our inventive plasma-hydrophilised surfaces. Efficient cell adhesion and growth could be accomplished for fibroblast-like cells on surfaces having water contact angels in the interval 10-40° and for epithelial like cells in the interval 5-40°.

30

## RESULTS

### Planar CD discs plasma-treated

Plasma Science PS0500 reactor with one electrode plate

Samples placed on a polypropylene support 20 cm from the reactor chamber floor in the center of the chamber

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Disc	Gas	Gas flow set	RF power	Power/flo	Plasma	Contact	Contact angle
material		value		w	time	angle direct	washed in
		sccm	W	W/sccm	Min	70% EtOH	
poly-carbonate	Oxygen	15	500.	33,33333	5	3	25
poly-carbonate	Oxygen	15	300	20	5	3	38
poly-carbonate	Oxygen	10	500	50	5	3	11
polycarbonate	Oxygen	10	300	30	5	4	31
poly-carbonate	Oxygen	5	500	100	5	3	5
polycarbonate	Oxygen	5	300	60	5	4	16
poly-carbonate	Air (synth)	25	500	20	5	4	17
poly-carbonate	Air (synth)	25	300	12	5	10	33
polycarbonate	Air (synth)	5	500	100	5	3	2
poly-carbonate	Air (synth)	5	300	60	5	4	13
Poly-carbonate	Argon	100	500	5	5	25	48
Poly-carbonate	Argon	100	300	3	5	27	56
Poly-carbonate	Argon	25	500	20	5	4	18
Poly-carbonate	Argon	25	300	12	5	9	39
Poly-carbonate	Argon	5	500	100	5	4	3
Poly-carbonate	Argon	5	300	60	5	4	9
Zeonex	Oxygen	100	500	5	5	20	29
Zeonex	Oxygen	100	300	3	5	17	34

24							
Zeonex	Oxygen	50	500	10	5	10	5
Zeonex	Oxygen	50	300	6	5	15	28
Zeonex	Oxygen	25	500	20	5	7	4
Zeonex	Oxygen	25	300	12	5	11	10
Zeonex	Oxygen	5	500	100	5	4	2
Zeonex	Oxygen	5	300	60	5	4	4
Zeonex	Air (synth)	100	500	5	5	16	29
Zeonex	Air (synth)	100	300	3	5	16	36
Zeonex	Air (synth)	50	500	10	5	9	6
Zeonex	Air (synth)	50	300	6	5	15	26
Zeonex	Air (synth)	25	500	20	5	5	4
Zeonex	Air (synth)	25	300	12	5	9	6
Zeonex	Air (synth)	5	500	100	5	8	4
Zeonex	Air (synth)	5	300	60	5	5	4
SAN	Oxygen	100	500	5	5	8	27
SAN	Oxygen	100	300	3	5	11	23
SAN	Oxygen	50	500	10	5	8	7
SAN	Oxygen	50	300	6	5	9	22
SAN	Oxygen	25	500	20	5	5	7
SAN	Oxygen	25	300	12	5	7	14
SAN	Oxygen	5	500	100	5	4	2
SAN	Oxygen	5	300	60	5	5	3
SAN	Air (synth)	100	500	5	5	8	27
SAN	Air (synth)	100	300	3	5	8	26
SAN	Air (synth)	50	500	10	5	7	8
SAN	Air (synth)	50	300	6	5	8	26
SAN	Air (synth)	25	500	20	5	4	5
SAN	Air (synth)	25	300	12	5	6	12
SAN	Air (synth)	5	500	100	5	5	4
SAN	Air (synth)	5	300	60	5	4	4
PMMA	Air (synth)	50	500	10	5	20	40
PMMA	Air (synth)	50	300	6	5	39	53
PMMA	Air (synth)	25	500	20	5	8	21
PMMA	Air (synth)	25	300	12	5	26	44
PMMA	Air (synth)	10	500	50	5	4	4
PMMA	Air (synth)	10	300	30	5	6	14
PMMA	Air (synth)	5	500	100	5	8	4

25

PMMA	Air (synth)	5	300	60	5	5	3
PMMA	Oxygen	50	500	10	5	29	54
PMMA	Oxygen	50	300	6	5	39	52
PMMA	Oxygen	25	500	20	5	11	40
PMMA	Oxygen	25	300	12	5	31	53
PMMA	Oxygen	10	500	50	5	5	10
PMMA	Oxygen	10	300	30	5	7	45
PMMA	Oxygen	5	500	100	5	4	4
PMMA	Oxygen	5	300	60	5	4	7

## C L A I M S

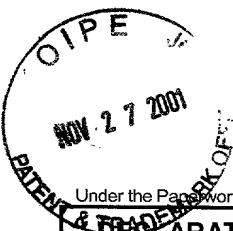
1. A method for rendering a substrate surface made in plastic material more hydrophilic by treatment in a gas plasma of a non-polymerizable gas, characterized in that the intensity of the plasma is selected so that the surface becomes permanently more hydrophilic, and with the optional step that the surface subsequently is washed with a pure solvent selected from water, a water-miscible solvent and mixtures thereof,  
5 said surface containing a liquid transportation system in which there is a channel with a depth  $\leq 1000\mu\text{m}$ .
2. The method according to claim 1, characterized in that  
15 the plasma intensity is  $\geq 5 \text{ W/cm}^3/\text{min}$ .
3. The method according to claim 2, characterized in that a power  $\geq 250 \text{ W}$  and a gas flow  $\leq 50 \text{ cm}^3/\text{min}$  are applied.
- 20 4. The method of any one of claims 1-3, characterized in that the plastic material has been selected among plastics having an immediate water-contact angle  $\geq 20^\circ$  and  
(a) the plasma treatment conditions are set so that the immediate water-contact angle after plasma treatment  
25 and a subsequent wash with pure water/ethanol (70 % w/w) becomes  $\leq 30^\circ$ .
5. The method of any one of claims 1-4, characterized in that the plastic material is selected among  
30 (a) polymers/copolymers in which the monomers are unsaturated such as (i) alkenes/alkadienes, and acids, esters, amides, nitriles etc containing one or more alkene groups; and (ii) vinyl aryl compounds in which the vinyl group is bound to aryl that optionally is substituted with lower alkyl groups (C1-6);  
35 (b) condensation polymers/copolymers in which the monomomers are selected from compounds exhibiting two

or more groups selected among amino, hydroxy, carboxy etc groups.

6. The method of any one of claims 1-5, characterized in that  
5 the plasma is induced by radio- and/or microwaves.
7. The method of any one of claims 1-6, characterized in that  
the plasma gas is oxygen, nitrogen, or a noble gas, such  
as argon, or a mixture of these gases.  
10
8. The method of any one of claims 1-7, characterized in that  
subsequent to the plasma treatment the surface of the  
substrate is derivatized to exhibit groups selected  
amongst anion exchanging groups, cation exchanging groups,  
15 amphoteric groups, hydroxy groups, bioaffinity groups, and  
chelating groups.
9. The method of any one of claims 1-8, characterized in that  
at least part of the surface comprises a liquid  
20 transportation system comprising a channel which has a  
depth  $\leq$  1000  $\mu\text{m}$ .
10. A substrate surface which is made of a plastic  
material, which has been plasma treated, characterized in  
25 that the surface in uncoated form has an immediate water-  
contact angle  $\leq$  30°, said water-contact angle being  
changed less than  $\pm$  20% and/or less than  $\pm$  5° upon washing  
with a pure ethanol/water mixture (70% w/w).  
30
11. The substrate of claim 10, characterized in that the  
plastic material is selected from  
(a) polymers/copolymers in which the monomers are  
unsaturated such as (i) alkenes/alkadienes , and  
acids, esters, amides, nitriles etc containing one or  
35 more alkene groups; and (ii) vinyl aryl compounds that  
optionally is substituted with lower alkyl groups (C1-  
6);

- (b) condensation polymers/copolymers in which the monomers are selected from compounds exhibiting two or more groups selected among amino, hydroxy, carboxy etc groups;
- 5 said polymer material optionally being cross-linked and/or optionally being a mixture of two or more kinds of polymers/copolymers.
12. The substrate of any one of claims 10-11, characterized in that the surface before having been gas plasma treated exhibits an immediate water-contact angel  $\geq 30^\circ$ .
- 10
13. The substrate of any one of claims 10-12, characterized in that at least part of the surface comprises a liquid transportation system comprising a channel which has a depth  $\leq 1000 \mu\text{m}$ .
- 15
14. The use of the substrate defined in any one of claims 10-13 and/or obtained by the method defined in any one of claims 1-9 for the culturing of cells.
- 20
15. A kit characterized in comprising
- (a) a microfabricated device comprising a substrate surface which is made in a plastic material and which comprises a liquid transportation system comprising a channel which has a depth  $\leq 1000 \mu\text{m}$ , and
- 25
- (b) a fluorescent substance to be detected in the device, the fluorescence intensity of the plastic material being  $< 50\%$  of the fluorescent intensity of the substance at the wavelength at which the substance fluoresces.
- 30
16. The kit of claim 15, characterized in that the plastic comprises a polymerisation product obtained by polymerisation of an aliphatic monomer in which there is unsaturation.
- 35

17. A method for culturing anchorage-dependent cells and non-anchorage dependent cells that in a part of their life cycle require attachment to a substrate surface, characterized in that the substrate surface is made in a plastic material and has an immediate water-contact angle  $\leq 30^\circ$ .
- 5
18. The method of claim 17, characterized in that the substrate surface has been obtained by the gas plasma treatment method defined in claims 1-9.
- 10
19. The method of any one of claims 17-18, with the proviso that for anchorage-dependent cells the substrate surface enables at least 30 % of the plated cells to adhere to 15 the substrate surface.
20. The method of any one of claims 17-19, characterized in that at most 15 % of the culture medium is serum.
- 20 21. The method of any one of claims 17-20, characterized in that culturing is taking place during a time period permitting the number of cells to be at least duplicated.
22. The method of any one of claims 17-21, characterized in that the cell culturing is carried out in a chamber of a microfabricated device in which there is a liquid transportation system comprising (a) a channel having a depth which is  $\leq 1000 \mu\text{m}$  and (b) said chamber which also provides said substrate surface.
- 25



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**DECLARATION (CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)**

As a below named inventor(s), I/we declare that:

The declaration is directed to:

 The attached application, or Application No. 09/937,533, filed on September 24, 2001 as amended on \_\_\_\_\_ (if applicable).

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT international filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

**FULL NAME(S) OF INVENTORS:**Inventor one: Anders LarssonSignature: Anders Larsson Citizen of: SwedenInventor two: Anette OcklindSignature: Anette Ocklind Citizen of: SwedenInventor three: Helene DerandSignature: Helene Derand Citizen of: Sweden

Inventor four: \_\_\_\_\_

Signature: \_\_\_\_\_ Citizen of: \_\_\_\_\_

 Additional inventors are being named on \_\_\_\_\_ additional form(s) attached hereto.**Declaration for Utility or Design Patent Application**

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EK102656049US, in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date shown below.

Dated: 11/27/01 Signature: Staci Harris (Staci Harris)